Online Control of *Lemna minor* L. Phytoremediation: Using pH to Minimize the Nitrogen Outlet Concentration

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CVD 800

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Dissertation submitted as a requirement for Master of Engineering.

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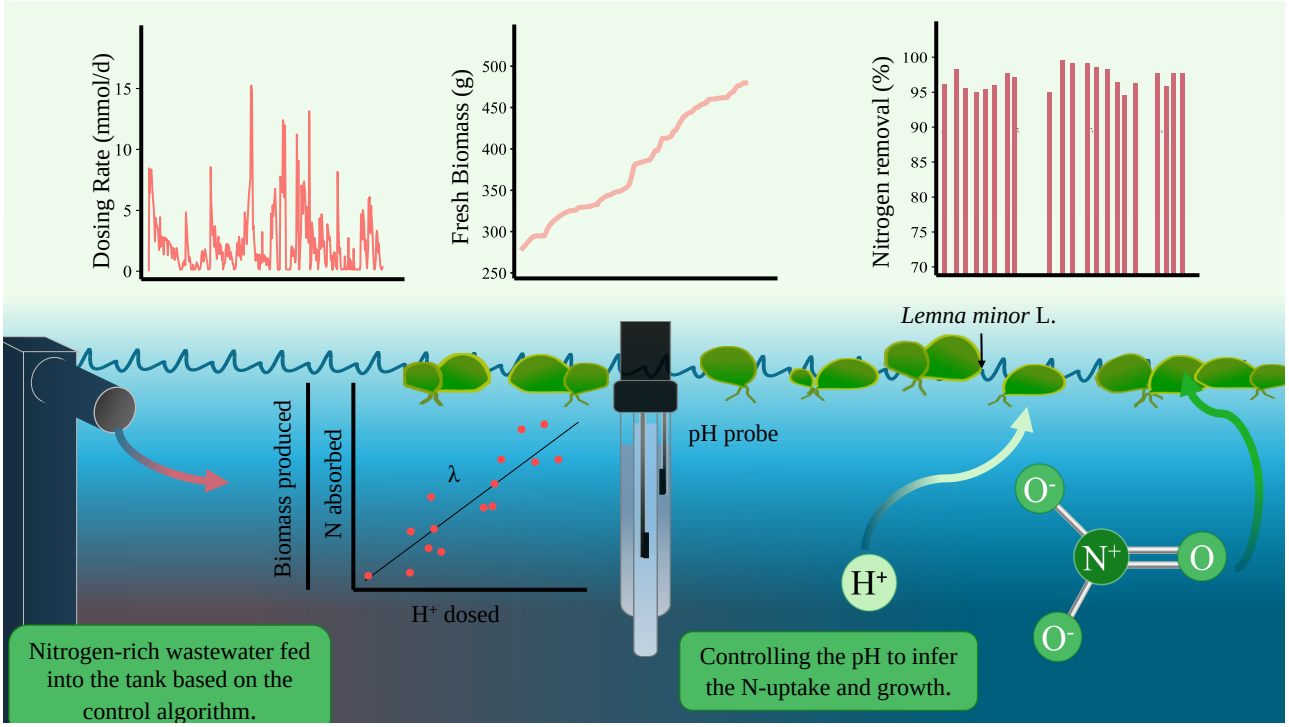
Abstract



Ecological remediation has gained significant attention recently due to the adverse ramifications of anthropogenic waste in water. For several years, natural processes have been incorporated in centralised wastewater treatment. Furthermore, natural wetland systems and constructed wetland systems are instances where aquatic ecosystems are able to facilitate high impact pollutant removal. As such, ecological phytoremediation technologies are employed worldwide to remove nutrient pollutants from agricultural and industrial wastewater. Unfortunately, standard process control methodology in phytoremediation systems has not been fully realised — a common rationale is that plant-based technologies have been limited for use in large open-air environments like the aforementioned wetlands, lagoons, and stabilisation ponds. It is understood that with adequate control system infrastructure, nutrient removal is greatly improved, as is the case with algae-based nutrient removal research. Notwithstanding consistently low outlet concentrations of nitrogen and phosphorous, control systems often involve the use of expensive analytical instruments and are therefore rarely viable. In the current study, *Lemna minor* (lesser duckweed) was

grown in 20 L batches of modified Hoagland's solution in de-ionised water with pH and level control capabilities. The results present a successful application of phytoremediation process control. Alkalisation of the liquid medium was observed in the pH as a response to the uptake of nitrate. The nitrate uptake was determined by standard spectrophotometric method. Despite the difference in biomass amounts, it was evident that a constant ratio existed between the amount of nitrate removed and the amount of acid dosed (required for pH control), which was equal to 1.25 mol N · (mol H^+)$^{-1}$. The pH response due to the co-absorption of NO_3^- and H^+ ions made it possible to use the pH measurement as the sole input to control the nitrate outlet concentration. A proportional-integral controller was used to maintain near-neutral pH of 6.5 in a continuously operated phytoremediation tank covered by *L. minor*. A nitrogen control strategy was developed which exploited this relationship between nitrate uptake and dosing and successfully removed upwards of 80 % of the fed nitrogen. At critically low nitrate concentrations (in the range of 0.05–0.3 mM), the nitrate to proton ratio was reduced to 1.08 mol N · (mol H^+)$^{-1}$. The biomass growth rate was successfully predicted based on the acid-dosing rate. This study demonstrates a clear illustration of how advanced chemical engineering control principles can be applied in phytoremediation processes.

Keywords: phytoremediation; nutrient pollution; pH control; nitrate removal; *Lemna minor*; nitrogen to proton ratio

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List of Nomenclature

N	elemental nitrogen
P	elemental phosphorus
NO_3^-, NO_3-N	nitrate ion, nitrate-nitrogen
NH_3, NH_4^-, NH_4-N	ammonia, ammonium, ammonium-nitrogen
N_2	molecular nitrogen
CW	constructed wetland
MWSP	macrophyte-based wastewater stabilisation pond
PO_4^{3-}	phosphate ion
K, K^+	potassium/potassium ion
RGR	relative growth rate (d^{-1})
Hoagland's solution	Hoagland's and Arnon (1938) growth medium with nitrate-nitrogen only
H^+	hydrogen ion/proton
HCl	hydrochloric acid solution (0.1 M concentration)
λ	nitrogen to proton ratio (mol N $\cdot$ mol^{-1} H^+)
Fresh weight	wet solid biomass (g)
C_{NO_3-N}	measured concentration of nitrate-nitrogen (mM or mmol $\cdot$ L^{-1})
ΔpH	absolute pH change
ΔpH_{avg}	running average of pH change based on ten values
$\Delta pH/\Delta pH_{avg}$	ratio of absolute pH change and average pH change
ϵ	ratio of pH-uptake reduction (pH units·pH units^{-1}

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Introduction

Eutrophication, air pollution, biodiversity loss, and climate change are just a few of the negative effects that nitrogen and phosphorus pollution from agricultural and industrial wastewater continue to have on the ecosystem — an issue which stringent policies and regulations are in place to circumvent (Speijers & Fawell, 2011; Chaturvedi, Chakraborty & Ardeep, 2020; Kanter & Chodos, 2020). Therefore, technologies such as reverse osmosis and chemical precipitation methods are employed to remove nitrogen and phosphorus from these wastewaters (Speijers *et al*, 2011). Biological methods such as constructed wetlands are considered to be ecologically friendlier and have gained increasing attention (Brix & Schierup, 1989; Helmer & Hespanhol, 1997). The use of aquatic macrophytes, such as *Lemna minor* L. (lesser duckweed), has been effective in lowering nitrogen and phosphorus concentrations to levels that are environmentally safe owing to fast pollutant removal rates and good process efficiency in these systems (Pescod, 1992; Lilley, Pybus & Power, 1997; Li *et al*, 2009; Li *et al*, 2010).

However, current trends in phytoremediation technologies indicate room for improvement. Constructed wetlands (CWs) and macrophyte-based wastewater stabilisation ponds (MWSP) are the two most common systems employed. CWs are commonly designed to facilitate phytoextraction of throughput at a certain flow rate by a polyculture of plants. Studies researching MWSPs tend to assess the use of plant monocultures for water treatment. Potential nitrogen removal efficiency by duckweed were reported to range between 50 % and 95 % for ammonium, upwards of 82 % for nitrate, and over 70 % for total nitrogen and ammonia, respectively while potential phosphate removal was reported to reach intermediate values of 30–55 % (Alaerts, Mahbubar & Kelderman, 1996; Körner & Vermaat, 1998; Benjawan, Lee & Koottatep, 2008; Su *et al*, 2019). Remediation with *Eichhornia crassipes* (water hyacinth), a close contemporary of duckweed-based systems, is known to have nitrate removal capability of up to 83 % (Ayyasamy *et al*, 2009; Rezania *et al*, 2016) and higher biological oxygen demand (BOD) reduction capability of up to 70 % (Körner *et al*, 1998; Fang *et al*, 2007).

The previously mentioned literature shows that phytoremediation has the potential to innovate water treatment technologies, however scaling of these technologies remain elusive; all of these results were obtained in batch systems. None of the literature surveyed provided information on how conventional phytoremediative methods have been adapted for continuous use. Thus, there appears to be an opportunity to apply standard process control principles in order to operate a continuous plant-based bioreactor — which would make it possible to link the online measurement of process variables to water quality amelioration and nutrient removal rate. Despite the advantages, measurement tools like ion-selective electrodes and advanced analytical techniques like elemental analysers are costly (Alford, 2006;

Lourenco *et al*, 2012; Salgado & Simões, 2013; Collos & Harrison, 2014; Gruiz & Fenyvesi, 2016).

Although online control of the phytoremediative water treatment systems remains highly conceptual, various studies have established the precedent for online measurement to control conditions such that optimal nutrient removal can be achieved. Within these, micro- and macroalgal treatment methods are common — the maximisation of biomass output is closely related to nutrient removal (Mcginn *et al*, 2017). In a study by Mcginn *et al* (2017), the biomass concentration, measured by in-house photometric method, was used as an input to control the outlet flowrate of a microalgae photo-bioreactor. In the method described by Franca *et al* (2021), the CO_2, NO_3, and total phosphorus concentrations were also inferred based on spectrophotometric measurements, however to control nitrogen and phosphorus concentrations, the CO_2 feed rate was manipulated. A major disadvantage of these systems is that they are suited to a very specific reactor configuration which makes use of inline spectrophotometry. As such, these methods are incompatible with plants commonly found in wetlands and open ponds, and thus cannot be adapted for use in simple systems.

The present study proposes that pH is a reliable and inexpensive measurement that could be used to provide a viable control option. When nitrate is absorbed and assimilated by plants, alkalisation of the aqueous medium occurs from the release of OH^- ions and the co-absorption of H^+ ions (Dijkshoorn, 1962; Hageman, 1984; Tischner, 2000; Cedergreen & Madsen, 2002; Tischner & Kaiser, 2007). Hence, pH can be used as the sole input variable for controlling the nitrate concentration in a phytoremediation system's discharge stream and therefore pH changes in the medium can be related to the amount of nitrate absorbed by *L. minor*. This would directly relate to the control of the nitrate concentration in the effluent of a *L. minor* water remediation tank. To test this a nitrogen remediation study was performed in a semi-continuously operated system, where the pH characteristics of the system were used to manipulate the input of synthetic wastewater. The relationship between growth, nitrate uptake and pH dynamics were established. The pH-nitrate-growth characteristics were incorporated in the feed algorithm of the semi-continuous system to control nitrate breakthrough and biomass removal. Ultimately, the wastewater throughput was manipulated as a function of the varying nitrogen removal characteristics of the pond.

Literature

2.1 Nitrogen in the environment

As an input to global food production, nitrogen is a commercially significant nutrient (Fields, 2004; Speijers *et al*, 2011; Ward *et al*, 2018; Kanter *et al*, 2020). However, the global average nitrogen use efficiency (NUE) is generally considered to be very low — NUE measures the degree to which crops utilise nitrogen to maximise yields. Hence, NUE indicates the maximum possible crop yield relative to the input used. Maximum NUE for developing nations are at 40–55 %, largely dependent on farming practices (Martínez-Dalmau, Berbel & Ordóñez-Fernández, 2021). While studies have found that nitrogen inputs above the optimal have little to no effect on the crop yield, significant amounts of nutrients accumulate in soil and eventually leak into nearby river and groundwater. Most of the nitrogen applied to crop plantations is inorganic reactive nitrogen which is unavailable to most other consumers aside from certain types of archaea and bacteria — with respect to crop production, excess nitrogen input goes unutilised. Hence, there is a need to balance optimal crop productivity with the potential harm to the environmental. The general view is that more work should be done to effectively regulate nutrient use without severely reducing crop outputs (Zhang *et al*, 2010; Panhwar *et al*, 2019).

Inorganic nitrogen is fundamental in forming proteins and amino acids required in all living things. Most of this nitrogen is unavailable to biological organisms as plants take up inorganic nitrogen from the soil (or water). Before the establishment of the modern fertiliser industry, most of the reactive nitrogen in the biosphere originated from the upper atmosphere. Consequently, innate processes of ancient simple organisms facilitated gaseous molecular nitrogen (N_2) fixation into the biosphere such as biological nitrogen fixation by nitrogenase-carrying prokaryotes (Pajares & Bohannan, 2016; Ward *et al*, 2018) which still exist in the present day. Furthermore, biological nitrogen fixation accounts for 45.6 % of global reactive nitrogen production relative to abiotic nitrogen fixation at 33.1 % (Boyer *et al*, 2004).When nitrogen is fixed, N_2 is converted into ammonia (NH_3). Within both free-living and symbiotic cells, the biochemical reduction of molecular nitrogen is energy intensive, with an ATP expenditure of 8 moles per mole NH_3 produced, catalysed by nitrogenase. The Haber-Bosch process was discovered in the early twentieth century and has since been the principal method for supplementing nitrogen and phosphorus (Ward *et al*, 2018). In contrast, hydrogen gas (H_2) is reacted with N_2 to produce NH_3 as a major component of synthetic fertiliser. Fields (2004) indicates that the contribution of fertiliser production accounts for 100 million–170 million megagrams of nitrogen fixated per year.

The nitrogen cycle refers to the continuous, uninhibited cycling of nitrogen between the at-

mosphere and biosphere. The term suggests that reactive nitrogen species do not amass and that the different nitrogen transformation processes allow self-regulation of the nitrogen balance — microbial consortia promote nitrogen fixation, ammonification, nitrification, and denitrification. Processes in the upper atmosphere also contribute to nitrogen fixation to a lesser extent (Pajares *et al*, 2016) however these processes will not be discussed further in this review. Nitrification naturally occurs aerobically by ammonia-oxidizing and nitrite-oxidizing prokaryotes. Nitrification is a two-phase oxidation process whereby ammonia is initially oxidised to nitrite (NO_2^-) and then subsequently to nitrate (NO_3^-). Ammonia is regenerated when organic nitrogen in tissues is degraded — a process known as ammonification — available for uptake by plants and other microbes. Denitrification releases gaseous nitrogen into the atmosphere. Under anaerobic conditions, denitrifying bacteria reduce NO_3^- to N_2 (Fields, 2004; Pajares *et al*, 2016).

Human-related reactive nitrogen inputs were shown to have a considerable impact on the global nitrogen cycle — this is due to the high potential of augmenting the amount of biologically available nitrogen in aquatic and terrestrial ecosystems. As a consequence of permanently altering the nitrogen regulation in the biosphere, excess soil nitrates and ammonia have been specifically linked to nitrogen leakage or leaching (Speijers *et al*, 2011; Chaturvedi *et al*, 2020) — becoming a potent source of surface water and groundwater pollution.

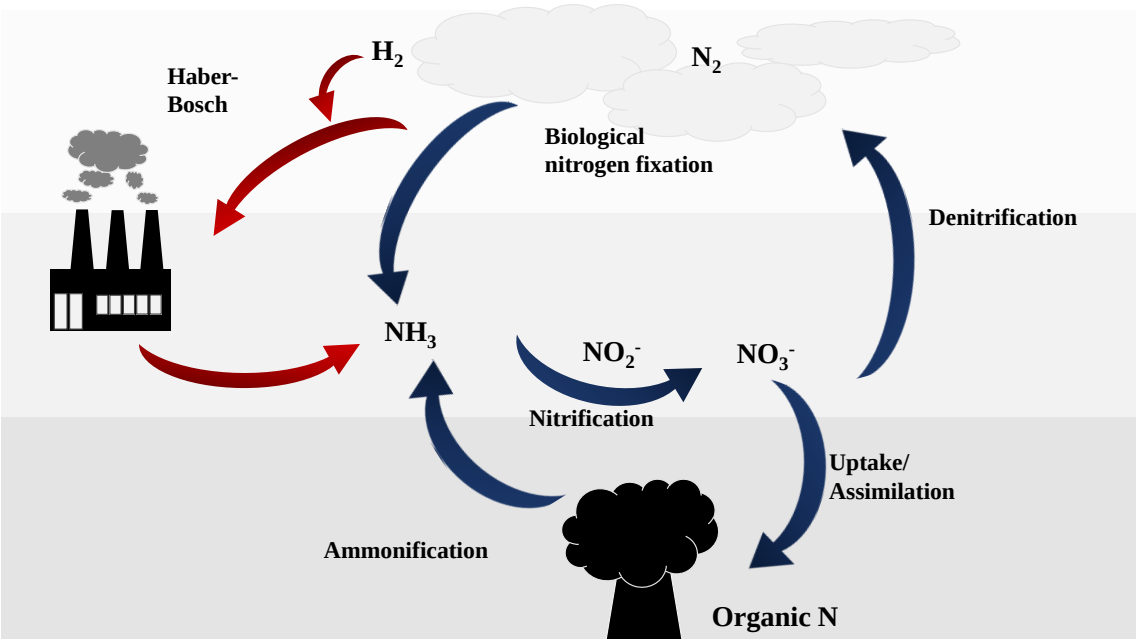

Figure 1: Relationship between different nitrogen species in the Nitrogen Cycle

2.1.1 Nitrogen uptake by plants

Nitrogen and carbon are required by plants in the highest amounts - while carbon is available in the air as carbon dioxide, nitrogen must be taken up from the growth medium (Bewley, 1981). Uptake is an active process and thus energy is required to take up nitrogen ions from the rhizosphere or root-zone medium through the cell boundary. A concentration gradient drives anion and cation uptake as reduction-oxidation reactions which maintains the balance of charges within the cell. Haynes (1990) described that the charge balance is maintained through the exchange of protons (H^+) and hydroxides (OH^-) with the extracellular environment. Tischner (2000) indicated that a proton-nitrate symport is largely responsible for nitrate-nitrogen absorption in both terrestrial and aquatic plants while Dijkshoorn (1962) and Haynes (1990) emphasise that nitrate uptake was attributed to a counter-transport excretion mechanism of OH^-.

Conclusively, the NO_3^-/H^+ symport and NO_3^-/OH^- antiport mechanisms have equivalent influences on the charge balance. It is understood that pH is an important indicator of the predominant nutrient uptake mechanism. Anion absorption is linked to an increase in medium pH (alkalisation). Conversely, cation uptake is associated with a decrease in the pH (acidification). In other words, alkalisation is observed in a plant-medium supplied with nitrate-nitrogen and acidification is observed in the medium of a plant grown in ammonia. These alkalisation and acidification effects have been shown to overcome the buffering capacity of a medium (Eisele & Ullrich, 1975; Tischner, 2000; Tischner *et al*, 2007).

Based on the author's observations, Eisele *et al* (1975) suggested that there was a stoichiometric relationship between the efflux of OH^- and relative uptake of nitrate. This was confirmed by Haynes (1990). Tischner (2000) made association between cation uptake (such as K^+, Mg^{2+}, Na^+, and Ca^{2+}) and nitrate uptake as alternate antiport nitrate uptake mechanisms (Eisele *et al*, 1975).

2.2 Impacts of nutrient pollution

As the world's population increases, so does the demand for synthetic fertiliser to meet the higher demand for food. The escalated use of synthetic fertilisers over the last few decades has been related to a swell in the nutrient pollution to the air and water (Kanter *et al*, 2020). Several investigations have been done to address the impacts of excessive nutrient leaching and suggest that it is a preferable approach to limiting supply-side nutrient loss (Lilley *et al*, 1997; Pereira *et al*, 2012; Fan *et al*, 2013; Epsztein *et al*, 2015; Chaturvedi *et al*, 2020). Moreover, limiting nitrogen and phosphorus inputs may not be feasible without causing a significant reaction in the food markets (Pajares *et al*, 2016).

Environmental policies have attempted to restrict waste discharge into rivers and coastal waterways. Nevertheless, rising nutrient concentrations render regulations almost moot. Wastewater effluent, sewerage, surface water runoff, stormwater runoff, and industrial discharge are recognised as drivers of nutrient pollution (Lofrano & Brown, 2010; Angelakis & Snyder, 2015; Ansari *et al*, 2015). The issue is that there is greater vulnerability in the essential supply of clean water to various water use and industries (Speijers *et al*, 2011; Ansari *et al*, 2015; Rout *et al*, 2021). Because of interactions with other negatively charged particles in soil, nitrates are more mobile than ammonia and very soluble in water (Tischner, 2000; Speijers *et al*, 2011). As a result, unassimilated nitrates tend to seep into groundwater and surface water runoff to a higher extent. It is estimated that normal surface water concentrations are around 10 $mg{\cdot}L^{-1}$ for nitrates, 5.0 $mg{\cdot}L^{-1}$ for ammonia, 0.1–1.0 $mg{\cdot}L^{-1}$ for phosphate and nitrite (Helmer *et al*, 1997; Speijers *et al*, 2011; WHO, 2011). These levels are regularly raised by discharge of untreated urban wastewater hence increasing the amount of reactive nitrogen. Many rivers and streams directly receive sewage not far from where people live. As the only available source of water, communities are vulnerable to serious illnesses. The World Health Organisation (WHO) highlight the common effects of excessive nitrate ingestion — the danger lies in that nitrate reduces to nitrite which impairs the capacity of haemoglobin to transport oxygen throughout the body. At high doses, methaemoglobinaemia has been observed in adults, however it is more common in children aged three to six months due to their smaller body weights (Speijers *et al*, 2011).

Nutrient pollution has far-reaching environmental implications in addition to lowering water quality. Brix *et al* (1989) and Ansari *et al* (2015) consider hypertrophic water to be a threat to biodiversity because endemic plant populations are unable to compete for resources with algae while animal population die due to anoxic conditions. Eutrophication is the process by which excessive nutrient enrichment encourages blue-green algae growth, converting potentially healthy aquatic into hypertrophic water. There is an observed decrease in dissolved oxygen (DO) as anaerobic decomposition of biomass persists at lower depths while surface diffusion of air is inhibited by surface greenification. Saprophytic bacterial populations increase as more decomposition takes place which is observed as an increase in the biological oxygen demand (BOD) (Brix *et al*, 1989; Brix, 1994; Lilley *et al*, 1997; Shrimali & Singh, 2001; Speijers *et al*, 2011; Pereira *et al*, 2012; Chaturvedi *et al*, 2020).

2.3 Nutrient Removal for water remediation

Nutrient reduction strategies have been in development for years. In wastewater treatment, ammonia and organic carbon are converted into less harmful forms by micro-organisms during the secondary treatment stage. Autotrophic bacteria such as ammonia-oxidising

bacteria (AOB) and nitrite-oxidising bacteria (NOB) facilitate the transformation of ammonia into nitrite and nitrite to nitrate, respectively. Heterotrophic bacteria are then used to anaerobically denitrify nitrate in the presence of sufficient carbonaceous material. Bioreactors that commonly apply the aforementioned nitrogen removal process are trickling filters, membrane bioreactors and continuous flow activated sludge systems (Lofrano *et al*, 2010; Angelakis *et al*, 2015). Non-biological technologies have been in use for several decades and have demonstrated comparatively good removal rates. Examples of these systems are reverse-osmosis (RO), electro-dialysis, ion exchange (IX), and chemical precipitation (Benjawan *et al*, 2008; Lofrano *et al*, 2010). In some cases, these technologies have been used in combination with one another.Table 1 briefly discusses the basic categories of removal methods.

Bioremediation has been identified as a less expensive alternative route than intensive physical or chemical treatment methods. A significant amount of research has been done with respect to plants' abilities to remediate hypertrophic water. Studies have shown that phototrophic organisms are remarkably efficient nutrient removal agents similar to traditionally used nitrifcation/denitrification systems. There are several advantages phototrophic remediation has over nitrification, one is that carboneous material is not required in significant amounts in wastewater. Additionally, waste nutrients can be recovered as algal or plant biomass

Phytoremediation has become an established technique to treat waste-polluted areas and have been found to be applicable for removal of both organic and inorganic pollutants (Benjawan *et al*, 2008; Su *et al*, 2019). The term originates from the combination of the Greek prefix *phyto* meaning plant and Latin suffix *remedium* meaning to restore and refers to the capability of plant-based technologies (natural or genetically engineered) to degrade or take up pollutant materials (Brix *et al*, 1989; Bhupinder, Sharmila & Saradhi, 2009). There are two recognised mechanisms through which contaminants are removed: direct uptake and storage of contaminants into plant biomass (only nutrients such as N and P will be assimilated into the biomass while phyto-toxic materials accumulate within the tissues); and stimulate rhizospheric microbial activity through exudation which facilitates bio-transformation of contaminant material to less harmful forms. The use of plants in heavy-metal removal is also well documented (Masinire, Adenuga & Tichapondwa, 2021).

Table 1: Categories of nitrate removal technologies commonly referred to in literature.

Standard removal methods	Working principle	Limitations	Reference
Physical methods			
Membrane technology	A semi-permeable membrane operated at moderate to high pressures allows water molecules to pass and rejects inorganic, organic NO_3^-.	A highly concentrated reject stream containing all removed nitrates is produced and must be properly disposed of. Pumps and pressurisation consume a lot of energy. Required to pre-treat and post-treat raw wastewater to reduce fouling Limited to small and moderate capacities due to cost and energy consumption	Epsztein *et al* (2015), Rout *et al* (2021)
Ion exchange	Strong basic anion substitution occurs within an inert matrix (resin) as water passes which takes up NO_3^- ion in exchange for Cl^- or HCO_3^- ions.	Effluent water must be managed correctly disposed of properly	Cliford & Liu (1993), Samatya *et al* (2006)
Biological methods			
Biological oxidation and denitrification of nitrogen	Ammonia-oxidising bacteria and reduction of NO_3^- to N_2 gas.	Relative to more advanced treatment technologies, biological nitrogen removal is much slower. Parameters like temperature and pH have huge influence on the rate of the process. To prevent growth inhibition, processes are required to remove metabolic products.	Reeves (1972), Rezania *et al* (2016)

2.4 Phytoremediation technologies

Without the influence of anthropogenic nutrient inputs, eutrophication is said to be a process that occurs over the period of years. Thus, aquatic systems are thought to self-remediate. In practice, phytoremediation technologies make use of natural processes to treat water and soil. Constructed wetlands (CWs), floating treatment wetlands (FTWs), and macrophyte-based stabilisation ponds (MBSPs) are examples of common phytoremediation technologies which are considered to be inexpensive solutions to treat wastewater (Greenway, 2003). CWs, MBSPs, and FTWs differ in a number of ways. According to literature, shallow water and slow retention rates are common, however there are distinctions based on the types of remediation processes utilised as well as the types of plants present, such as emergent, submerged, or free-floating plants CWs potentially remove up to 99 % and 96 % of COD and BOD respectively while total nitrogen removal is in the range of 90 % (Fan *et al*, 2013). Many examples illustrate how different variations on the basic designs of phytoremediation systems have been used to improve pollutant removal. The removal mechanism can be selectively influenced by the direction of flow for example in vertical flow wetlands and horizontal flow wetlands, while operating multiple wetlands simultaneously similar to multiple continuously-stirred reactors (CSTRs) in series has been found to increase the efficacy of phosphorus and nitrogen removal (Lilley *et al*, 1997).

The energy consumption, construction costs, and operating costs of CWs are reported to be inexpensive compared to contemporary technologies (Zhang *et al*, 2012; Wu *et al*, 2015). Despite the fact that several studies have established loading limits and frequently well-characterize wastewater inputs (Ayyasamy *et al*, 2009; Rezania *et al*, 2016), systems such as CWs and MBSPs have been suggested to be able to handle fluctuation in wastewater qualities. As a result, phytoremediation technologies have gained a substantial amount of popularity in recent years. There are still barriers in the way of widespread implementation. Because they require a large amount of space to work feasibly, there may be a bottleneck to decentralised wetland usage owing to a shortage of appropriate land. This is an issue that MBSP systems have been able to circumvent (Greenway, 2003; Fan *et al*, 2013; Wu *et al*, 2015).

Traditional wastewater treatment systems employ waste stabilisation ponds that do not make considerations for the recycling of key nutrients contained in wastewater. They have been associated with bad odours and poor nitrogen removal (FAO, 2000; Zimmo, 2003). Introducing aquatic plants provides a more convenient method to enhance waste stabilisation ponds and might treat nitrogenous wastewater at a lower cost. This appears to be the case in macrophyte-based ponds. Researchers have been assessing the use of aquatic plants, particularly duckweed, to purify agricultural and urban wastewater for the past sixty years (Hillman, 1961; Hillman & Culley, 1978; Zirschky & Reed, 1988; Alaerts *et al*, 1996; Van Der Steen, Brenner & Oron, 1998; FAO, 2000; Gupta & Prakash, 2013).

Plant uptake accounts for approximately 18–40 % of nutrient removal in duckweed-based stabilisation ponds, wetlands, and lagoons (Alaerts *et al*, 1996; Körner *et al*, 1998; Van Der Steen *et al*, 1998; Zimmo, 2003; Benjawan & Koottatep, 2007). Körner *et al* (1998) reported removal rates by duckweed as high as 95 % for ammonium and nitrate removal of greater than 82 %. Total Kjeldahl nitrogen and ammonia reduction in wastewater leachate amounted to over 70 % and 90 % respectively according to Alaerts *et al* (1996). Collaborative systems of aquatic plants have traditional been used in lagoons and ponds. A polyculture of *L. minor* and *Pistia stratiotes* (water lettuce) had the potential removal of 68 % of all nitrate and slightly lower total nitrogen removal capability compared to 55–63.2 % removal with *L. minor* monoculture (Benjawan *et al*, 2008; Su *et al*, 2019). As such, aquatic systems and similar remediation methods have been utilised to stabilise nutrient buildup in aquatic environments. By harvesting these aquatic plants, nitrogen and phosphate are eliminated from the system. Greenway (2003) attributes high removal rates to rapid biomass growth. Successful pilot projects have established the usage of aquatic plant ponds as functional units in wastewater treatment, yielding treated effluent with water quality comparable to that of sophisticated secondary treatment. MBSPs can be easily integrated into secondary and tertiary treatment systems, facilitating nutrient removal. The water quality in duckweed-based treatment ponds in particular is such that the effluent is considered suitable for reuse in fish pond aquaculture and crop irrigation (Rose *et al*, 1999). Duckweed wastewater treatment systems have demonstrated nitrogen removal efficiency ranging between 50–60 % (Boniardi *et al*, 1994) and 63–99 % (Körner *et al*, 1998).

2.5 Duckweed-based systems

Duckweed is a small plant with high potential for use in emerging photoremediative technologies. These plants are free-floating macrophytes that are established on the surfaces of water bodies. Duckweed presents interesting value-added applications for a bio-based fertiliser, high-protein animal feed and bio-energy feedstock owing to high starch content

(up to 45 %) (Liu *et al*, 2020), high protein content (36.0–45.0 %) (Gupta *et al*, 2013), and low lignin content (about 12 %) (Yadav *et al*, 2017). As such, many duckweed species are frequently exploited in waste nutrient recovery systems (Alaerts *et al*, 1996; Körner *et al*, 1998; Bhupinder *et al*, 2009), among which *L. minor* is one of the most widely distributed.

The literature describes the variances in duckweed growth patterns in the wild and how such differences are driven by environmental variables such as seasonal temperature fluctuations and changes in light during the day and night cycle (Alaerts *et al*, 1996; Landesman *et al*, 2005; Lasfar *et al*, 2006; Ansari *et al*, 2020). As such, the relative growth rates (*RGR*) are reported to be influenced by abiotic variables such as nutrient concentrations, temperature, mat density, photoperiod and light intensity (Lasfar *et al*, 2006). Lasfar *et al* (2006) determined that optimal nitrogen and phosphorus concentrations for a maximum RGR of greater than 0.40 d^{-1} at 40 $mg{\cdot}L^{-1}$ and 15 $mg{\cdot}L^{-1}$, respectively. When the concentrations rose above the optimal concentrations, the relative growth rate decreased significantly. The same observation was made for temperatures greater than and lower than 25 °C, which was determined to be the optimal growth temperature in Lasfar *et al* (2006).

Lasfar *et al* (2006) found a relationship between the photoperiod and optimal growth rate. Lasfar *et al* (2006) showed that a photoperiod of 12 h resulted in the fastest growth rate, however this partly conflicted with the findings of Yin *et al* (2015). Yin *et al* (2015) observed that a photoperiod of 16 h was related to 10–20 % higher starch production in the initial 3–4 days in *Lemna aequinoctialis* compared to the same species grown under the same light intensity (20–400 $\mu mol \cdot m^{-2} \cdot s^{-1}$) using a 12 h photoperiod. Additionally showed that maximum duckweed production at a 16 h photoperiod was significantly higher than duckweed growth using a photoperiod of 12 h for all light intensities tested except for 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The study also found that the biomass productivity of duckweed samples was greater at higher light intensities at both 12 h and 16 h photoperiods, however moderately lower light intensities (110 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were preferred by *L. aequinoctialis* using a 24 h photoperiod. This implied that longer photoperiods allowed for greater biomass yields, except in the case when the light intensity was too high, thus the a greater yield was achieved at relatively shorter photoperiods using higher light intensities. The results of Lasfar *et al* (2006) at a 24 h photoperiod may indicate a potential metabolic fatigue or growth inhibition owing to a shorter dark/night phase.

Duckweeds have the capacity to form dense mats on the surface of water bodies. This has led to the belief that mat thickness has a non-negligible impact on growth rate (Alaerts *et al*, 1996; Monette *et al*, 2006). Authors have reported optimal values of mat densities which vary greatly; the values were included in Table 2. Monette *et al* (2006) devised a harvest methodology to ensure regulated eutrophication at an appropriate mat density. An optimal mat density occurred when maximum duckweed productivity was reached at a specific

harvest rate. At the optimal mat density, duckweed mats were able to stop the formation of microalgae in the medium (Monette *et al*, 2006). According to Monette *et al* (2006), very high mat densities potentially limit the availability of resources such as air, light, and nutrients to duckweed plants in the layers beneath the water surface. This phenomenon was shown to reduce both the rate of growth and the rate of nitrogen removal (Lasfar *et al*, 2006; Monette *et al*, 2006). The relative growth rate was shown to reduce by three times for duckweed mats of 750–1200 g wet biomass $\cdot$ m^{-2} compared to smaller mats which were 3–18 times less dense (Monette *et al*, 2006). In spite of this, Alaerts *et al* (1996) found that larger lagoon systems were able to maintain aerobic regions which were necessary for natural aerobic processes.

Table 2: Optimal mat density published by literature

Mat density (g·m^2 wet mass)	**Reference**
>1600	Alaerts, Mahbubar & Kelderman (1996)
1250	Koles, Petrell & Bagnall (1987)
400–800	Skillicorn, Spira & Journey (1993)
750	Monette *et al* (2006)
45	Kahlert & Loderer (2020)

pH is one of the most critical environmental parameters influencing chemical uptake and distribution in living plants. Haynes (1990) write that taking up nutrients involves the release of counterions from the plant cell, which has an effect on the pH as more exudate material is released into the surroundings. Tischner (2000) discusses the characteristic pH change as nitrogen is taken up and assimilated. During nitrogen assimilation, nitrates are converted into ammonium ions releasing a hydroxide ion (OH^-). Mclay (1976) indicates that high pH above 10 affects nitrogen-availability. At ideal pHs of between 5 and 8, ammonium does not readily reduce to ammonia which cannot be taken up by plants.

Table 3: Relative growth rates (*RGR*) associated with duckweed such as *L. gibba* and *L. minor*

Medium	***RGR* (d^{-1})**	**Species**	**Reference**
Hutner	0.30	*L. gibba*	Greenberg, Huang & Dixon (1992)
Hutner	0.29–0.31	*L. gibba*	Mkandawire & Dudel (2005)
33 % strength Hutner	0.45	*L. minor*	Blaser *et al* (2008)
50 % strength Hutner	0.26	*L. minor*	Brooks, Riley & Taylor (2006)
Steinberg	0.21–0.61	*L. minor*	Drost, Matzke & Backhaus (2007)
Modified Steinberg	0.32	*L. minor*	Naumann, Eberius & Appenroth (2007)
Moifeid Johnsons	0.15	*L. gibba*	Mkandawire & Dudel (2005)

Several standard nutrient media associated with duckweed research have been formulated.

References to Pirson and Seidel's medium, Steinberg's medium, Hutner's medium, Johnson's medium, and Hoagland's medium have regularly been made in literature. The compositions of nutrient media were included in Table 4. Low strength formulas are often used such as Johnson's medium and Hutner's medium. The nitrogen and phosphorus requirements for optimal growth appears to be significantly lower than that of larger vascular plants for which Hoagland's medium was formulated. Iqbal (2019) highlights that more phosphorus is taken up as a fraction of the total weight for *L. minor* than for any other plant. However, this was still small relative to the nitrogen amount. In fact, nitrogen is identified as the limiting nutrient and is taken up to the largest degree by most plants (Tischner, 2000). Ericsson, Larsson & Tillberg (1982) found that growth rates could be affected by strictly controlling the nitrogen supply under nitrogen-limitation. Thus as nutrients are available *L. minor* will grow. Growth rates associated with *L. minor* in particular growth media have been presented in Table 3.

Table 4: Nutrient compositions of common growth media in literature

Nutrient	**Literature medium**				
	Hoagland & Arnon (1938) (mM)	Steinberg (1946) (mM)	Hutner's medium (mM)	Pirson (1950) (mM)	Johnson *et al* (1968) (mM)
N	14.99	5.96	0.5	4.35	0.01
P	1	0.73	0.23	2.61	0
K	6.01	4.93	0.46	5.41	0.03
Ca	4.99	1.25	0.08	0.85	0
Mg	2	0.41	0.2	3.41	0
S	2	0.41	0.23	3.41	0.01
Fe	0.05	0	0.01	0.05	0
Cu	0	0	0	0	0
Zn	0	0	0.02	0	0
Mn	0.01	0	0.02	0	0.01
B	0.05	0	0.02	0	0
Cl	0.02	0.01	0.2	8.77	0.03
Mo	0	0	0.01	0	0
Na	0.05	0	0.02	0	0
Co	0	0	0	0	0
Ni	0	0	0	0	0

Experimental

3.1 Experimental phytoremediation tank

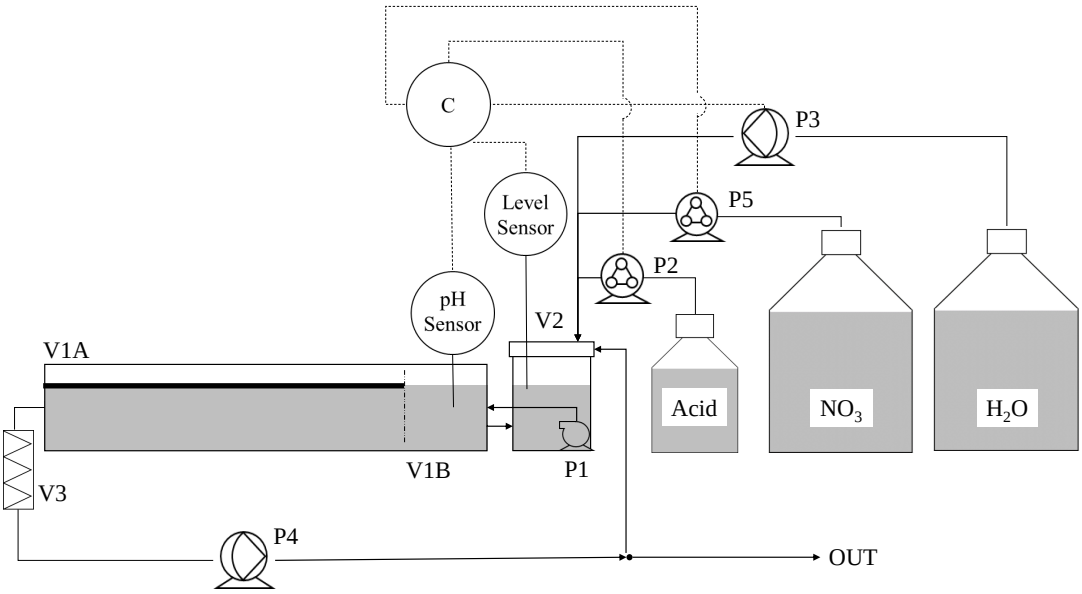

Figure 2: Phytoremediation tank experimental setup

The phytoremediation tank (rectangular tank with dimensions 80 cm X 51 cm X 9.8 cm) was built as per Figure 2. Shown in the figure, V1 was the main vessel with a total capacity of 35 L which was filled only to 20 L during experiments. *L. minor* was grown on the liquid surface in one of the two compartments, V1A. Plants were grown in an area of 0.27 m^2. V1A was open to the atmosphere; the compartment was lit by an overhead lamp. V1B was covered and separated from V1A to keep the compartment free of biomass. The pH of the vessel was measured in this compartment. V2 was a recirculation vessel which enables mixing of the fluids in the tank. Liquid was transferred to V2 from V1 by diffusion through a sponge filter and returned by pump P1. Acid was fed into V2 by peristaltic pump P2 in order to control the pH and NO_3-containing medium was fed into V2 by peristaltic pump P5. Level control instructed diaphragm pump P3 to feed de-ionised water into V2. Evaporation was the cause of water-losses. When operated continuously, diaphragm pump P4 was activated to discharge water from the tank. When operated in batch, the outlet line was not used. V3 was a filter that removed any suspended solid matter in the outlet as to not damage P4.

3.2 Apparatus and materials

A modified hydroponic medium *circa* 10 % dosage of full Hoagland's medium Hoagland & Arnon, 1938 was prepared for all batch runs (Runs 1–3), composed of 50.5 mg· L^{-1} KNO_3, 118 mg· L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 123.25 mg· L^{-1} $MgSO_4 \cdot 7H_2O$, 13.6 mg· L^{-1} KH_2PO_4, 2.25 mg· L^{-1} Fe-NaEDTA, 0.286 mg· L^{-1} H_3BO_3, 0.008 mg· L^{-1} $CuSO_4 \cdot 5H2O$, 0.181 mg· L^{-1} $MnCl_2 \cdot 4H_2O$, 0.022 mg· L^{-1} $ZnSO_4 \cdot 7H_2O$, and 0.012 mg · L^{-1} $NaMoO_4 \cdot 2H_2O$. For continuous runs (Runs 4–6), the medium was composed of 123.25 mg· L^{-1} $MgSO_4 \cdot 7H_2O$, 13.6 mg· L^{-} KH_2PO_4, 2.25 mg·L^{-1} Fe Na-EDTA, 0.286 mg·L^{-1} H_3BO_3, 0.008 mg·L^{-1} $CuSO_4 \cdot 5H_2O$, 0.181 mg·L^{-1} $MnCl_2 \cdot 4H_2O$, 0.022 mg·L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.012 mg·L^{-1} $NaMoO_4 \cdot 2H_2O$ and 13.48 mg·L^{-1} KNO_3, 31.487 mg·L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ as well as 197.09 mg· L^{-1} KCl and 388.81 mg· L^{-1} $CaCl_2 \cdot 2H_2O$. Chemical materials used to make up the synthetic media were sourced from Merck™/Sigma Aldrich™ (purity of 98%). In Run 6, the aforementioned medium was used with an additional 71.82 mg· L^{-1} hydrogen peroxide (2.11 mM) for algal control in the medium supply.

Level control and pH control were facilitated by an Arduino MEGA 2560™ microcontroller. Measurements were taken using an Analog Haoshi H-101 pH meter pro and logged regularly. The pH was adjusted with the addition of 0.1 M hydrochloric acid solution using a stepper motor peristaltic pump. Water and tank purge was done using a Kamoer™ 12 V diaphragm pump. For the liquid medium, chemicals were sourced from Merck™/Sigma Aldrich™ (purity of 98%). MarsHydro hydroponic lights provided the lighting for the tank.

3.3 Preparation of biomass

Non-axenic *L. minor* culture was obtained from the botanical gardens and greenhouses at the University of Pretoria (S 25° 45' 21" E 28° 13' 51"). Plants were cultured in-house on modified hydroponic growth solution in 20 L and 40 L basins under lighting with photoperiod of 16 h several weeks before the inoculation of the next run. The biomass was rinsed prior to inoculation in de-ionised water.

3.4 Visual analysis method

A high-definition web-camera (Ausdom® 1080p HD web camera, model: AW635, resolution: 1920p X 1080p) was fixed to a position above the surface of the phytoremediation tank (V1A). The web-camera was used to capture a still image of *L. minor* in the tank. The images taken from the web-camera were feed into a mass quantification algorithm. Prior to

the run, the algorithm was calibrated with images of the biomass in the phytoremediation tank where the masses were known.

The image data was analysed within a Python programme — this programme primarily made use of the OpenCV library necessary for image processing. The images were read by the programme and were reduced into smaller images with a resolution of 540 pixels by 540 pixels. The images were converted into a matrix wherein the RGB values (colour quantification system, representing the amount of red, green and blue) and the position values for each pixel in the reduced image were contained. The image processing involved the conversion of the RGB values into HSV values (another colour quantification system, representing the hue or colour, colour saturation or shade, and value or the brightness). K-means cluster counting method (MacQueen, 1967) was used to calculate coverage of the green objects in a particular image. This selected the pixels with HSV values within a desired colour range. The colour range was set to include all hues of green that were possible for the living duckweed in the lab. All the green pixels (which represented the "green" objects within the image) were identified and the other pixels that had colours outside the desired colour range were masked or hidden. The output of the Python programme was the total fraction of green pixels in a particular image relative to the total amount of pixels in the image. This output was converted into a mass value (grams of fresh biomass).

3.5 Analytical methods

Nitrogen in the liquid samples was analysed. Due to nitrate being used as the only nitrogen source, only nitrate analysis based on the photometric method was conducted: Spectroquant 0.10-25.0 mg/L NO_3-N Nitrate Cell Test and Spectroquant 23–225 mg/L NO_3-N Nitrate Cell Test from Merck^{TM}. Nitrate values were reported as the averages of three repeat tests on the same sample. Sampled values were calibrated for 340 nm wavelength spectrophotometer measurements (Agilent Technologies, Cary 60 UV-Vis, G66860A).

Biomass measurements included both fresh and dry weight measurements. Prior to inoculation, plants were rinsed and allowed dried for 45–60 min on paper towels before being weighed. At the end of a run, plants were carefully removed from the tank, dried and weighed to obtain fresh biomass. After drying for 48 h, the dry biomass weight was obtained. Representative fresh weight measurements were taken using a modified flat fishing net with known dimensions (70 mm X 92 mm). The relative growth rate (*RGR*) was determined using Equation 1 below:

$$RGR = \frac{\ln(\frac{M_f}{M_0})}{t_f - t_0} \tag{1}$$

where M_0 and M_f are the measured fresh weight (wet mass) at inoculation and after final removal in grams, and t_0 and t_f are the times of inoculation and final removal in days. The nitrogen removal in the effluent was reported for the remediation experiment in Run 6. Equation 2 was used to determine the removal of nitrogen. Inlet and outlet flow rates were the same.

$$\text{Fraction Removed} = \frac{F_{N_{fed}} - F_{N_{measured}}}{F_{Nfed}}, \tag{2}$$

where $F_{N_{fed}}$ is the molar flow rate of nitrate-nitrogen fed into the tank in mmol·d^{-1} and $F_{N_{measured}}$ is the molar flow rate of nitrate-nitrogen measured in the liquid medium in mmol·d^{-1}.

3.6 Methods and planning

Phytoremediation tank was inoculated with *L. minor* that was grown on 10 % Hoagland's medium with 16 h photo-period. The water level was reduced by evaporation and restored by addition of de-ionised water (level control). For all experimental runs, pH liquid environment was measured continuously and controlled. Abiotic conditions of the experiments such as light intensity and temperature were constant. Water temperature was measured throughout the duration of the batch (thermometer) and continuous runs (water sensor) and found to be the same as the air temperature. Regular measurements showed a slight deviation from the mean temperature of 22 °C by maximum of only 2 °C.

A photoperiod of 16 h was used for the batch runs as well as the stock culture of *L minor*. A modified hydroponic medium *circa* 10 % dosage of full Hoagland's medium Hoagland *et al*, 1938 was prepared. Run 1 and Run 2 were inoculated with duckweed such that the tank was only partially covered with biomass. At partial coverage, analysis of images taken was used to quantify the biomass in the tank. The amount of green colour that appeared in aerial image of the tank surface analysed using a K-means cluster counting method MacQueen, 1967. The imaging measurement was used as a comparison to the acid dosing based measurement. Run 1 was inoculated with 11.65 g, with a surface area coverage of 23.4 %. Run 2 was inoculated with 26.9 g, with surface area coverage of 61.2 %. Growth of *L. minor* at partial surface area coverage was compared to the growth at visibly full surface area coverage. In Run 3, 57.24 g of biomass inoculated the tank, where the tank's biomass density was measured via regular physical representative measurements.

Subsequent experiments testing the removal efficiency of a nitrogen removal strategy which was meant to control the nitrogen concentration in the tank effluent. The same starting biomass amount of 282 g for Run 4, Run 5 and Run 6. The pH controller was used to infer when nitrogen levels were low. For consistent and easier control, the photo-period was extended to 24 h and a 9400 lux lamp was used supplied light. The hydroponic medium used was 10 % dosage of full Hoagland's medium except for nitrogen which was lower than normal tenth dosage. Macro- and micro-nutrients were replenished by small doses of 300 % strength Hoagland's medium.

Results and discussion

4.1 Relationship between the nitrate uptake and proton dosing

In order to ascertain whether there was a significant relationship between proton dosing rate and nitrogen uptake rate, all three runs shown were performed in batch using 10 % Hoagland's medium. The pH in Run 1, Run 2 and Run 3 was controlled at a setpoint of 6.5. In these experiments, the hydrochloric acid dosing rate (D_R) was used as an indicator of the vegetative growth rate whereas true biomass measurements were used to obtain the *RGR* which assumed exponential growth. Despite the differences in the starting biomass, the growth rates were similar between the three runs presented, see Table 5. In trial runs, the *RGR* was in the range of 0.11–0.20 d^{-1} while in the tank, *RGR* remained consistently at 0.11–0.15d^{-1}. A main deduction that could be made based on experimental data was that there was a weak correlation between the inoculation biomass amount and growth rate. It was necessary to reconsider true growth data. Acid dosing revealed that growth initially displayed weak exponential behaviour, however after several days in the medium, there was a reduction in the growth rate as nutrients were near exhaustion. This calls into question the accuracy of *RGR*, as Equation 1 assumes purely exponential behaviour. A reason for this non-ideal behaviour was likely due to a change in the buffering capacity — buffering capacity was reduced which causes more frequent fluctuations pH and D_R as less acid was required to restore the pH setpoint. This behaviour was observed in Figure 7 (a) and Figure 7 (b).

Table 5: Initial biomass and final biomass weights for Run 1, Run 2 and Run 3 (batch).

	Initial (g)	Final (g)	*RGR* (d^{-1})
Run 1	11.65	65.97	0.11
Run 2	26.90	119.5	0.112
Run 3	57.24	177.9	0.125

Nitrate-nitrogen measurements confirmed that the nitrogen was reduced linearly in the tank; this was independent of the mat thickness or nutrient concentration. As a result, the nitrate consumption rate was found to be a constant value for all three runs. The relationship between the dosing rate and nitrate uptake rate was given in Figure 3, the plots of the nitrate uptake and the proton dosing for Run 1–3 appeared to lie on the same linear slope (λ = 1.25 mol N · (mol H^+)$^{-1}$). Therefore, for every mole of HCl dosed, 1.25 moles of nitrate was absorbed. With this link established, the proton dosing rate (*DR*) was able to provide information about the nitrate demand. There is a release of OH^- ions when NO_3^- is assimilated. The pH of the medium surrounding the roots rises as a result (Dijkshoorn, 1962; Hageman,

1984; Tischner, 2000; Tischner *et al*, 2007). It was assumed OH^- exudation per nitrate assimilated occurred at fixed ratio. The biomass production rate was then calculated by multiplying the proton dosing rate with λ and dividing by the nitrogen content in the biomass, which was measured at 61.9 mg N $\cdot$ g^{-1} dry mass. The water content of the duckweed was measured at 0.902 g $\cdot$ g^{-1}.

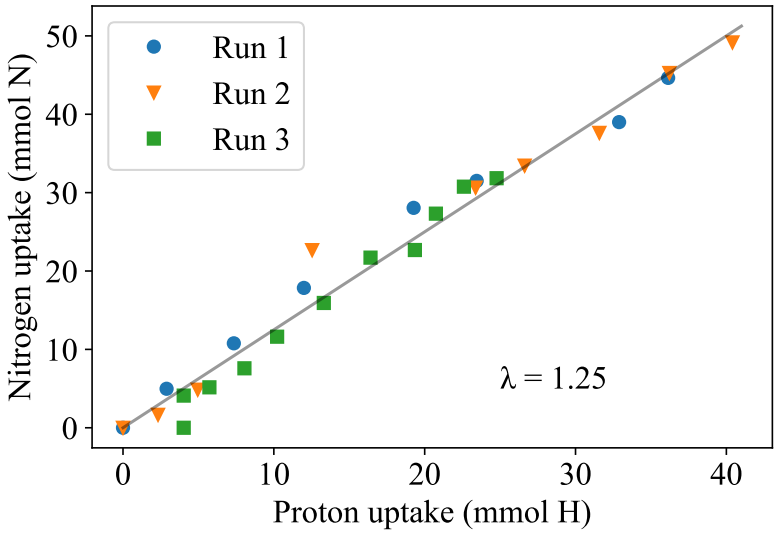

Figure 3: Absorbed molar nitrogen as a function of dosed protons

4.2 Approaches to biomass quantification

It is well known that *L. minor* has a vegetative growth pattern similar to bacteria. Daughter fronds are produced from the division of a mother frond. Duckweed biomass is thought to increase in two ways. When partially covered, large open spaces exist between clusters of fronds. Growth in this regime was associated with an increase in the surface area coverage until completely covered. The mat thickness would increase only after the liquid surface had been completely covered by biomass. This was referred to be a fully-covered regime. It was anticipated that no increase in biomass mat thickness occurred during partial coverage, and that once fully covered, there was no change in the amount of surface area coverage.

Runs 1 and 2 were inoculated such that the tank was partially covered with biomass. Run 1 was inoculated with 11.65 g, with a surface area coverage of 23.4 %, and stopped when the tank was completely saturated. Run 2 was inoculated with 26.9 g, with surface area coverage of 61.2 %. Run 2 was not terminated at full coverage and allowed to grow until nitrates were exhausted. In Run 3, 57.24 g of biomass were initially introduced, and the tank's biomass density was measured frequently. The distribution of fronds in the tank was measured using an image analysis method, which also served as a comparative measurement of the biomass

at partial coverage. The visual analytical technique measured the proportion of green pixels to all other pixels in overhead photographs of the liquid surface. Physical measurements were believed to be a reliable way to estimate the thickness across the entire tank.

A comparison of visual-based quantification and acid dosing-based quantification was done at partial coverage, as demonstrated in Figure 4 (a) and Figure 4 (b). The visual biomass quantification approach was a satisfactory comparison to the dosing-based quantification in Figure 4 (a) and appeared to be the most accurate of the two methods. This was especially true when the visual prediction occurred within the calibration limits and the output visual analysis algorithm did not seem to be limited by the movement or displacement of biomass fronds. Run 2 demonstrated that the visual technique was insufficient for identifying development above the tank surface's full capacity (Figure 4 (b)). Growth at partial coverage appeared to cutoff at 50–53 g and when compared to the dosing based quantification in the same figure, it was the least accurate of the two prediction methods. The dosing-based quantification more closely followed a reasonable growth trend until nitrate extinction. The curve inflection in Figure 4 (b) indicated that the maximum growth rate happened after 6 days. As a result, at higher mat densities, the repeatability of visual-based prediction was limited. It was required to re-adjust the calibration and extrapolate the visual estimate because growth had exceeded maximum capacity. Any significant mismatch between the visual quantification and dosage estimate can be ascribed to interference from algal infection. Growth beyond 200 $g{\cdot}m^{-1}$ (49—52 g) caused fronds to overlap, making gains in duckweed mat density imperceptible. This included any additional biomass that was not apparent, such as the roots that grew longer.

Since the visual quantification was unable to reliably estimate submerged biomass at complete coverage, the acid dosing-based quantification was compared to the measured mat density, as shown in Figure 4 (c). As per Figure 4 (c), mat density was not uniform across the entire surface of the tank and over-predicted the initial biomass gains. Accuracy grew as the biomass density in the tank increased. As a comparative quantification approach, it is difficult to argue that a representative mat density measurement was more valuable than just measuring total biomass repeatedly but this too has drawbacks, such as non-negligible mass losses. The representative method was only useful when the tank was extremely thick. Thus, Figure 4 (c) showed that acid dosing-based estimate was a reasonable predictor of biomass at greater mat densities.

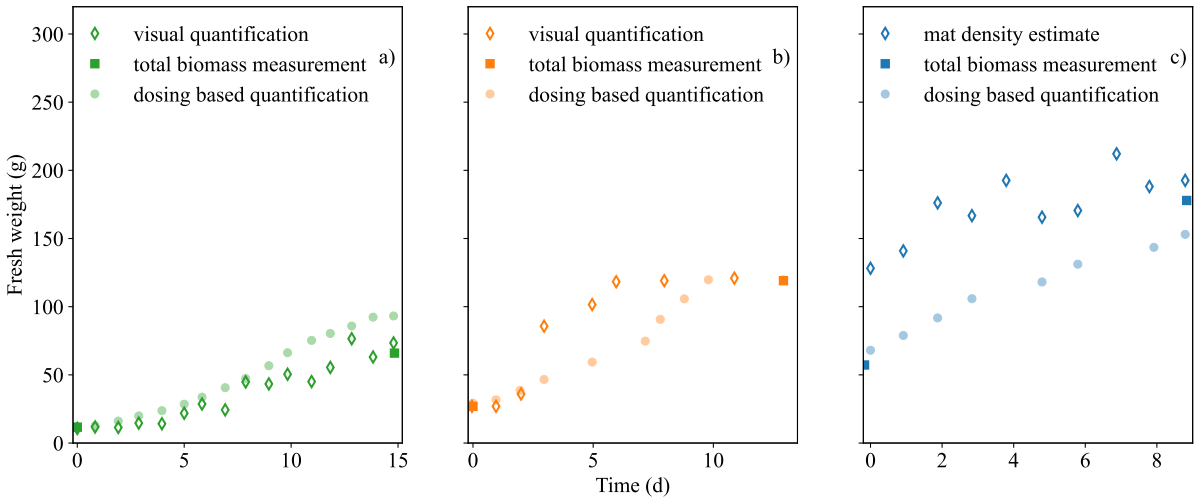

Figure 4: Comparison of visual based estimation (photo pixel analysis) of the biomass coverage in (a) Run 1 and (b) Run 2, biomass density determinations in (c) Run 3, and acid dosing based biomass quantification based in (a), (b) and (c)

4.3 Dosing-based algorithm for reducing nitrogen effluent in semi-continuous operation

The control method is depicted in Figure5. The pH control was based on detecting the pH between a rising and descending slope. Every thirty minutes, pH was sampled. A descending slope between two pH measurements was induced by an acid addition proportionate to the difference between the observed and setpoint pH. There were no acid additions between two pH measurements on the ascending slope, and thus the pH change was due to nitrate uptake. The ratio of the absolute pH difference on the rising slope and the average pH change ($\Delta pH/\Delta pHavg$) was computed and used as a requirement for identifying when to replenish the nitrogen supply. The average pH change was calculated using a running average of ten pH maximum readings, updated every hour. When administering nitrogen to the vessel, the running average was not computed. When nitrogen was depleted, pump P3 was activated to supply into the tank. To avoid misleading indicators, the control would only function if the previously measured pH maxima were reduced by more than 10 % (between a decreasing slope and rising slope). In order to treat nutrient-polluted water, the control algorithm above was implemented to act based on nitrogen-related pH behaviour, such that the phytoremediation tank was run semi-continuously. The method was based on the work of Van Rooyen & Nicol (2021).

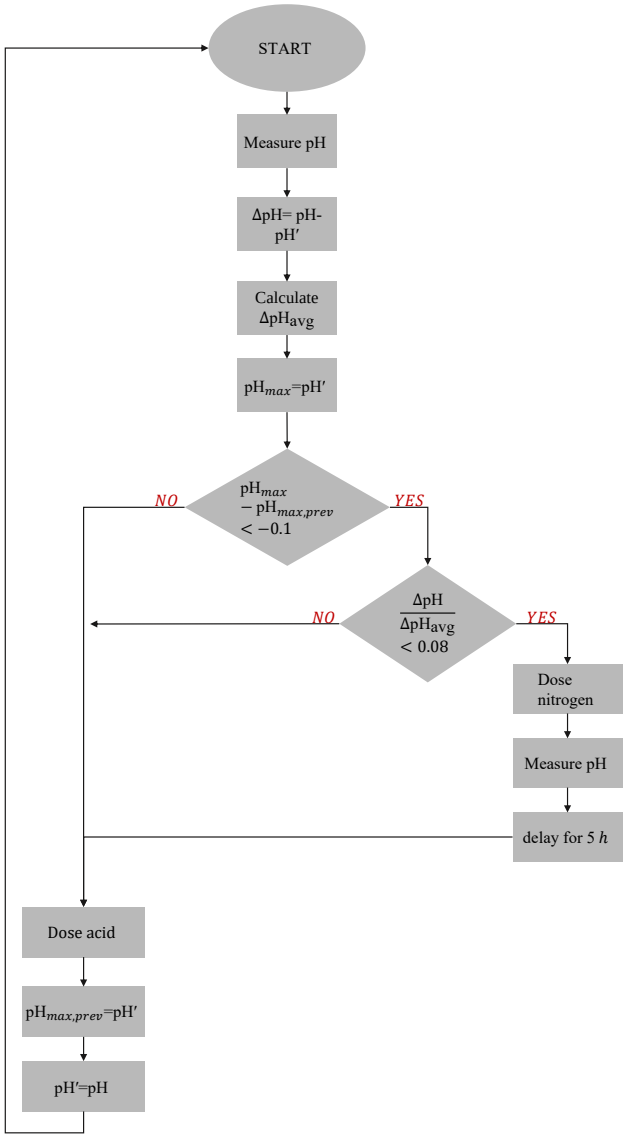

Figure 5: Control algorithm flowchart for detection of low nitrogen concentration

4.4 Significance of the dosing threshold

Exploratory implementation in Run 4 and Run 5 was shown in Figure 6, where $\Delta pH/\Delta pH_{avg}$ and nitrogen measurements were plotted for both runs. In the figure, nitrogen depletion was indicated by gray vertical lines. Nitrogen removal was tested at two distinct settings for the depletion threshold (ϵ), which determined how sensitive the controller was: 0.08 and 0.20. $\Delta pH/\Delta pH_{avg}$ had decreased below 0.20 (Figure 6 (a)) and 0.08 (Figure 6 (b)), the controller identified that as the characteristic response at for nitrate exhaustion.

When $\Delta pH/\Delta pH_{avg}$ fell below ϵ, nitrogen was no longer at a detectable concentration for the plant. The nitrate-nitrogen concentrations were seen to decrease with time until a minimum nitrate level was attained, 0.22 mM in (a) and 0.061 mM in (b) medium nitrate concentration despite regular feeding of fresh medium dosed at the times indicated on the plot. This showed that there was a significant difference in the effluent nitrogen concentration at ϵ of 0.2 compared to ϵ of 0.08. Due to the higher ϵ in Figure 6 (a) than in Figure 6 (b), the time between depletion instances was shorter.

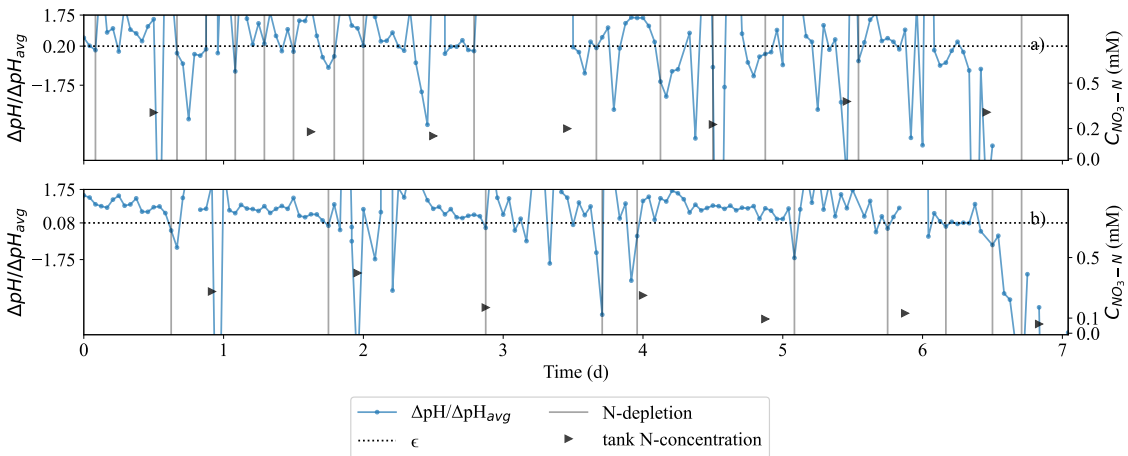

Figure 6: Comparative plot of $\Delta pH/\Delta pH_{avg}$ and nitrate-nitrogen concentration reaching depletion. The sensitivity/depletion threshold (ϵ) were: (a) 20 % and (b) 8 %.

Van Rooyen and Nicol (2021) explained that $\Delta pH/\Delta pH_{avg}$ approached zero as more nitrates were consumed by *Brassica oleracea* which showed that effective control could be achieved at desired nitrate concentrations to maintain a healthy growing environment. It was believed that the same stringent control may be used to maintain extremely low nitrate. The effluent could be kept at a very low nitrogen concentration — minimisation control with an ϵ of 0.08 was shown to be superior than minimisation control with a ϵ of 0.2. This was the situation in Run 5 (Figure 6 (b)), where the nitrogen in the tank effluent could be regulated between 0.05 mM and 0.15 mM significantly lower than in Run 4 (Figure 6 (a)). At ϵ of 0.08, remediation obtained extremely low nitrogen concentrations, as shown in Figure 7 (c); concentrations at various depletion occurrences were nearly zero. This is further reinforced by

a rapid drop in pH, which resulted in a decrease in D_R. This is thought to have coincided to the reduction in exudation noted by Dijkshoorn (1962) and Tischner (2000) as responsible for alkalisation of the liquid. It was understood that the lower rate of alkalisation in the medium implied that the growth was slower.

On account that the phytoremediation system was operated non-sterilely, it was undisrupted by any bacterial infections. This meant that no work was done to sterilise the duckweed biomass before inoculation (beyond using hydrogen peroxide to prevent algal growth). Therefore, the system was solely vulnerable to algal bloom. Algal growth could be inhibited at ideal biomass quantities. An algal infestation would develop in the open areas where the water's surface was not covered by vegetation (if biomass density was lower than 200 $g \cdot m^{-2}$). Algae, like duckweed, would alkalise the growing medium; nevertheless, the alkalisation effect caused by algal bloom was discovered to alter the pH by 0.001–0.005 pH units $\cdot$ h^{-1}. This was regarded insignificant compared to the contribution of *L. minor*. Algal growth was kept under control by recirculating the tank medium (the line at pump P4), and completely covering the tank with *L. minor*. Therefore, 286 g was selected as the initial biomass. Every second day, duckweed was harvested. This was done to guarantee near-constant biomass coverage.

4.5 Implementing a continuous nitrate removal control system

In Run 6, the tank was run continuously for 21 days. After inoculation, the duckweed was allowed to grow in the medium for the first 110 hours of the experiment to acclimate the plants after transfer until the initial nitrogen depletion at time zero in Figure 7. When all available nitrogen was depleted, the feedback proportional-integral controller instructed the pump, P5 (Figure 2), to supply fresh 10 % Hoagland's medium to restore all nutrients. $\Delta pH/\Delta pH_{avg}$ was plotted in Figure 7 (a) and D_R was reported in Figure 7 (b). The sharp decreases in $\Delta pH/\Delta pH_{avg}$ corresponded to nitrate depletion in Figure 7 identified after the pH slopes had decreased 92 % relative to the running average. In Figure 7 (c), the nitrate concentration was measured to be very low while in Figure 7 (d), the associated nitrate removal was calculated. The figures should be understood as follows: of the nitrates fed into the reactor, the nitrate removal shows the fraction of nitrates eliminated from the throughput. The remediation system had an average throughput rate of 7.2 $L \cdot d^{-1}$ and a retention time of 2.96 days.

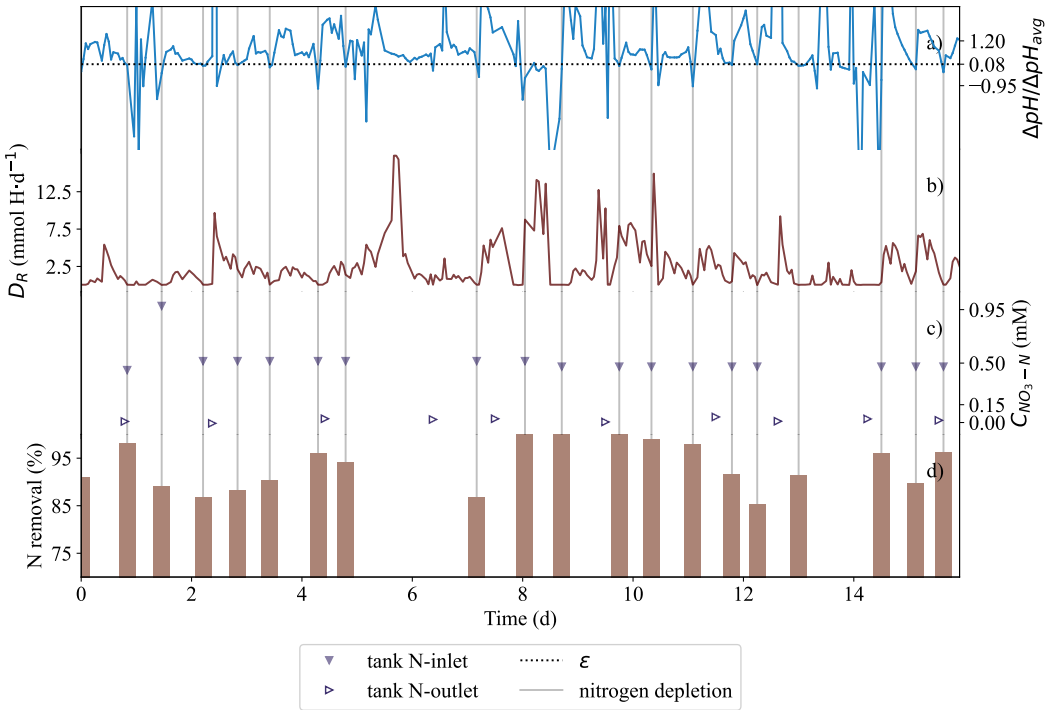

Figure 7: Full phytoremediation semi-continuous run: (a) $\Delta pH/\Delta pH_{avg}$; (b) D_R; (c) Effluent C_{NO_3-N}; (d) Percentage fraction nitrogen removal

4.6 Dosing-based biomass quantification measuring nitrogen removal

Total biomass amounted to 474.66 g, representing a 65.96 The total nitrate nitrogen removed from the liquid was found to be 59.39 mg NO_3-N·g^{-1} dry biomass, whereas the nitrogen removed by the biomass was estimated to be 61.90 mg NO_3-N·g^{-1} dry biomass. Furthermore, it was demonstrated that biomass in the continuous system could be estimated, in the same manner as the dosing based prediction for the batch runs (Figure 4. In Figure 8 (a), a λ-value of 1.08 mol N · (mol $H^+)^{-1}$ was calculated as a better fit for the new biomass growth in Figure 8 (b) depicts the forecast. The difference between the estimated dosage and the measured biomass was 2.37 %.

A prediction error of 2.37 % confirmed that the dosing biomass prediction method was sufficiently accurate. Nitrogen removal could be quantified in terms of biomass production (59.39 mg NO_3-N · g^{-1} dry biomass nitrate removal compared to an estimated change in biomass nitrogen of 61.90 mg NO_3-N · g^{-1} dry biomass). The biomass increase was over-predicted, and the prediction error was substantially more than that of what was presented in Figure 8 (a) when the former λ of 1.25 mol N · (mol $H^+)^{-1}$ was used. Hence, the nitrogen to proton ratio was recalculated for Run 6 (λ = 1.08 mol N · (mol $H^+)^{-1}$). There was a decrease in λ between the batch and the continuous systems which could not be attributed to the shortage of any other macronutrient (except for nitrogen, all other nutrients were given

in excess). Comparatively, the controller dosed more protons than nitrates that were taken up. The nitrate uptake was likely affected by nitrate availability. Although, it is currently unknown to what extent the other nutrient ions (calcium, magnesium, potassium, phosphate, and sulphate) contributed to λ in both in nitrate-sufficient and nitrate-limited conditions, it is believed that nitrate had the largest affect on λ (Haynes, 1990; Tischner, 2000). The author surmises that the sluggish growth of *L. minor* was related to the reduction in λ.

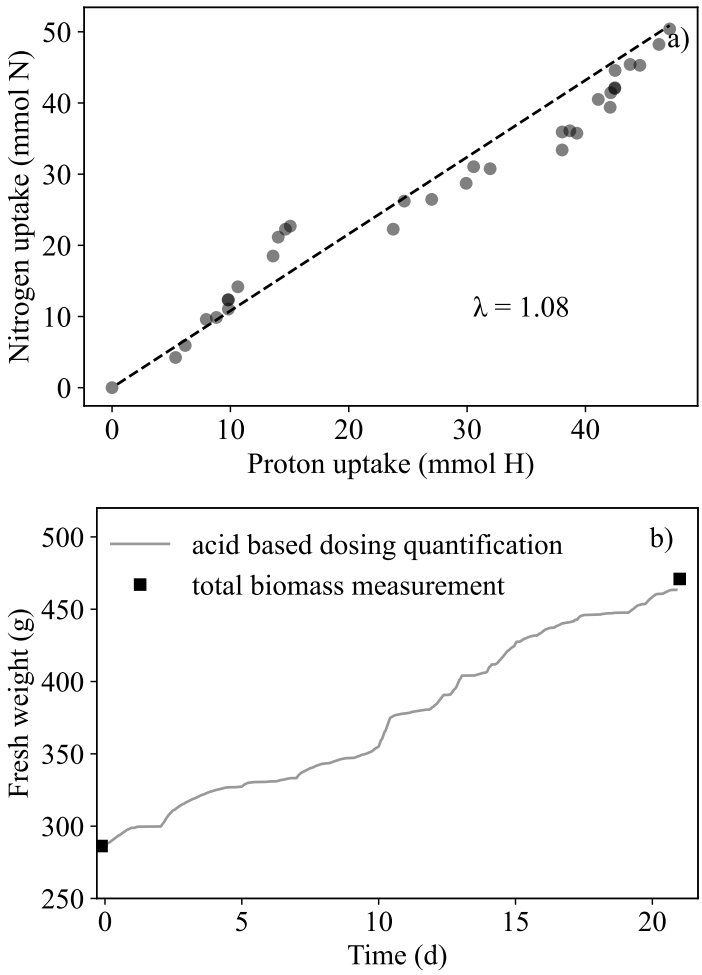

Figure 8: (a) The relationship between absorbed nitrogen and dosed protons (λ) and (b) acid-dosing based biomass prediction in the continuous run.

4.7 The trade off between high nitrate removal and growing speed in an automated nitrogen removal system

Nitrate depletion was detected approximately every 10–14 h. In Figure 7 (c), nitrogen concentration in the tank varied between 0.0 mM and 0.30 mM with an inlet nitrogen feed concentration ranging between 0.5 mM and 1 mM. Due to the system operating until depletion, the treated water effluent could be discharged at low outlet concentration. Therefore

the removal of nitrate was dependent on *L. minor's* uptake characteristics. The 80 % nitrate removal efficiency was an indicator of good performance in the system despite a slow growth rate. This result is comparable to that of Körner *et al* (1998), Alaerts *et al* (1996), and Ayyasamy *et al* (2009). Nitrate measurements and nitrogen removal data in Figure 7 (d) confirmed that disruptions at 5–7 days did not effect the efficiency of removal severely.

There seemed to be a trade-off between the high nitrate removal and the growth rate. Under nutrient sufficient conditions, *L. minor* is able to grow relatively fast as indicated by Bian *et al* (2012). There appears to be a minimum nitrogen concentration such that the growth rate is optimum. The medium contained an initial concentration of 0.40 mM NO_3^- while traditional nutrient media have a nitrogen concentrations of between 5 mM to 15 mM. Although the system was able to operate under nitrogen-lean conditions, it was observed that there was slow biomass growth which was likely a stress response to the nitrogen. As such, it could be said that high nitrogen removal efficiency was prioritised at the cost of fast biomass growth. As previously discussed, one could infer this observation from the dosing rate in Figure 7 (b). There was a sharp decrease in D_R as the system approached nitrate depletion. This resulted in short periods of zero to very little dosing. As soon as the medium was fed, dosing increased again. Appenroth, Augsten & Mohr (1992) suggests that a physiological dormant response of *Spirodela polyrhiza* was positively associated with low nitrate concentrations and that turion germination could be stimulated by the presence of nitrate. As such, low D_R was probably an indicator of sluggish activity, however this was not examined in depth.

An observed increase of 188.66 g in the biomass was measured which corresponded to an increase of 65.96 % of the inoculation mass over the course of 21 days. Under normal conditions, this would be considered very slow production. The relative growth rate of 0.017 d^{-1} was ten times lower than in previous batches (Table 5). Higher growth rates have been associated with nutrient removal (Benjawan *et al*, 2008; Li *et al*, 2009; Li *et al*, 2010; Collos *et al*, 2014; Mcginn *et al*, 2017; Chaturvedi *et al*, 2020). Ultimately, the lower yield could be considered a consequence of the nitrogen-limitation stress. At such a low nitrogen supply, this was to be expected. The stock culture of *L. minor* was prepared with nitrogen supply of 1.5 mM NO_3^- while nitrogen in the tank varied between 0.01 mM to 0.3 mM.

It was also observed that the rate of nitrate uptake increased as compared to just after inoculation. Within the first 100 h after inoculation, the absorption rate of NO_3^--N was found to be 0.0144 $mmol \cdot g^{-1} \cdot d^{-1}$. After 16.5 d, the rate had increased to 0.0927 $mmol \cdot g^{-1} \cdot d^{-1}$ which demonstrates a higher demand for nitrogen, possibly due to nitrogen-lean stress mechanisms of duckweed. Root growth was also observed in Figure 9. It was thought that the development of dense roots from fronds occurred as physiological response to nitrogen limitation thus affecting the uptake rate. Cedergreen *et al* (2002) noted their observation of root growth of duckweed having a linear proportionality to the ammonium and nitrate uptake

rate. Although the literature referenced in the study (Appenroth *et al*, 1992; Cedergreen *et al*, 2002; Landesman *et al*, 2005; Appenroth *et al*, 2018) mention that the carbon to nitrogen ratio within the biomass tended to increase, the present study cannot say whether there was a change to the elemental composition within the biomass, further work is required to confirm this.

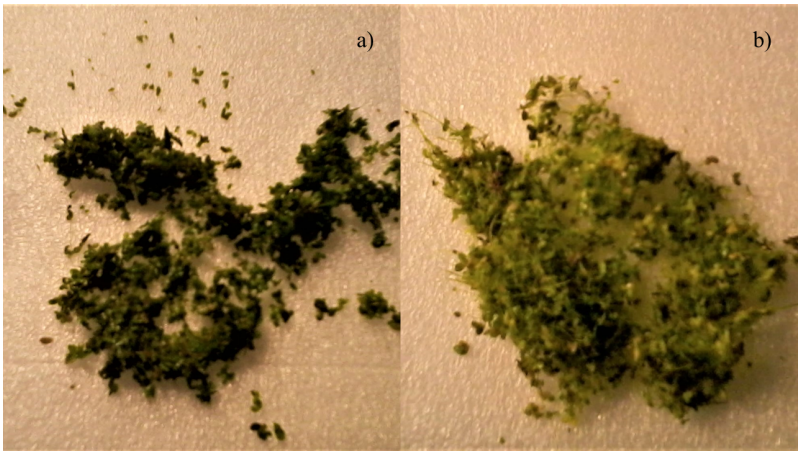

Figure 9: Images of *L. minor* from (a) before the run, and (b) after the run.

4.8 Limitations

Unlike the ideal conditions in the system demonstrated with the synthetic medium used in the study, wastewater is usually characterised to contain both ammonium-nitrogen and nitrate-nitrogen (Angelakis *et al*, 2015). The pH control algorithm presented above is able to specifically identify the removal of nitrate-nitrogen. The reason for this is ammonium-nitrogen would be taken up by the duckweed, however the system would require an alkali solution to raise the pH. This is due to the positive NH_4^+ ion; the charge balance would result in the absorption of OH^+ which would lower the pH in the vessel, assuming there is more ammonium-nitrate than nitrate-nitrogen on a molar basis. One could amend the system to feed medium at ammonium-depletion and control the pH using NaOH. This was attempted in an exploratory experiment and was feasible to implement. However, the pH control is currently limited to one nitrogen species due to both nitrate and ammonium affecting the pH differently. This would make it difficult to identify which nitrogen species was required without measuring the concentration because the depletion response of one nitrogen species would be characterised as the same as the uptake response of the other nitrogen species. Consequently, the system is currently only conceptual.

Conclusion

The work demonstrates the validity of the proposed hypothesis that by simply using pH as an input it was possible to remove nitrogen from wastewater in a continuous phytoremediation system. After quantifying the pH-nitrate-growth characteristics, duckweed growth was inferred based on the nitrogen uptake. The acid dosing most accurately quantified biomass regardless of whether duckweed partially covered or completely covered the surface and was a non-destructive, online measurement of biomass. For that reason, dosing was a better measurement strategy than measuring the biomass mat density or estimating biomass visually. The study demonstrated a unique method of nutrient removal from water using *L. minor*. The nitrate-nitrogen concentration in effluent was controlled using a proportional-integral feedback control scheme by using the pH as an input variable. This was attributed to the system's capacity to discharge water as soon as it identified nitrogen depletion. This resulted in a sufficiently high throughput of treated water of 7.2 $L{\cdot}d^{-1}$ and high nitrogen removal rates of more than 80 %. It was found that a high nitrogen removal was obtained at the cost of growth as *RGR* showed a decrease of 90 %. In a larger system it would be necessary to consider environmental factors, namely temperature (influenced by seasonal changes) and lighting (influenced by the time of day) as well as mixing which may impair the accuracy and repeatability of pH and nitrate readings. The project was entirely conceptual and consequently there is no direct implementation as of yet.

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